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# Influence of Inorganic Phosphate on Photosynthesis of Wheat Chloroplasts

## II. RIBULOSE BISPHOSPHATE CARBOXYLASE ACTIVITY

F. MÄCHLER AND J. NÖSBERGER

*Institut für Pflanzenbau, ETH-Zentrum, Universitätstrasse 2, CH-8092 Zürich, Switzerland*

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## ABSTRACT

Isolated wheat chloroplasts were pre-incubated in the dark in the presence of various concentrations of inorganic phosphate with or without carbon dioxide, oxaloacetate, glycerate, and 3-phosphoglycerate. The effect of subsequent illumination on photosynthetic oxygen evolution, ribulose bisphosphate carboxylase activity, ATP content, and ribulose bisphosphate content was investigated. Inorganic phosphate had little effect on ribulose bisphosphate carboxylase activity in darkness or during the initial phase of illumination, but it prevented the decline in activity that occurred during later stages of illumination, when photoreduction of $CO_2$ was decreasing in rate. Addition of inorganic phosphate to chloroplasts illuminated without phosphate restored the ribulose bisphosphate carboxylase activity, increased the ATP, and decreased the ribulose bisphosphate in the organelles. The responses to $CO_2$, oxaloacetate, glycerate, and 3-phosphoglycerate suggest that the decreased activity of ribulose bisphosphate carboxylase during photosynthesis results from ATP consumption.

Purified ribulose bisphosphate carboxylase was activated by inorganic phosphate, but this activation did not occur in the presence of ATP. ATP inhibited ribulose bisphosphate carboxylase when it was present in combination with various photosynthetic metabolites.

Inactivation of ribulose bisphosphate carboxylase in chloroplasts, illuminated in the absence of inorganic phosphate, is not due to lack of activation by inorganic phosphate or ATP. It may result from decreased stromal pH.

Key words: Ribulose bisphosphate carboxylase; Chloroplasts; Wheat; Phosphate; ATP.

## INTRODUCTION

Ribulose bisphosphate carboxylase (RuBPCO) activity in chloroplasts is low in the dark and increases upon illumination (Sicher, 1982). Light activation may be attributed to an increase in stromal pH and $Mg^{++}$ concentration (Werdan, Heldt, and Milovancev, 1975; Lorimer, Badger, and Andrews, 1976). Activity of RuBPCO in chloroplasts was high upon illumination for 8–10 min and then declined in a similar way to the rate of $CO_2$ fixation, suggesting that the decline in $CO_2$ fixation may have been caused by deactivation of the enzyme. However, the addition of 3-phosphoglycerate or glycerate doubled the initial rate of $CO_2$ fixation without increasing the RuBPCO activity (Stumpf and Jensen, 1982). The authors speculate that the

Abbreviations: ATP: adenosine triphosphate; $P_i$: inorganic phosphate; RuBP: ribulose bisphosphate; RuBPCO: ribulose bisphosphate carboxylase.

lack of correlation in the observed rates of photosynthetic $CO_2$ fixation and the initial activity of the RuBPCO is due to a discrepancy between measured and actual RuBPCO activity.

The present study investigates the cause for the decline of RuBPCO activity during photosynthesis. It may be the result of the consumption of $PO_4^{3-}$ ($P_i$), since this is an activator of RuBPCO (Bhagwat, 1981). Furthermore, Heldt, Chon, and Lorimer (1978) suggest that $P_i$ is a prerequisite for the light activation of RuBPCO in chloroplasts. The present study investigates four problems. (1) RuBPCO activity in illuminated chloroplasts, with and without $P_i$ in the medium, is compared. (2) The influence of the $P_i$ concentration in the medium on the contents of ATP and ribulose bisphosphate (RuBP) in the chloroplasts is investigated. (3) RuBPCO activity during the photoreduction of various substrates differing in their consumption of ATP and $P_i$ is investigated (4). The inconsistency between the response of purified RuBPCO to $P_i$ and of RuBPCO in chloroplasts to $P_i$ is studied by measuring the activity of purified RuBPCO in the presence of ATP, $P_i$, and other metabolites.

## MATERIALS AND METHODS

### *Chloroplast experiments*

Plants were grown, chloroplasts were prepared and photosynthesis was measured as described earlier (Mächler, Schnyder, and Nösberger, 1984). Experiments were conducted in an oxygen electrode at a photon flux density of 300 $\mu$mol $m^{-2}$ $s^{-1}$. Temperature was kept at 5 °C or 25 °C using a circulating water bath. To measure RuBPCO activity, 50 $mm^3$ samples of chloroplast suspensions were mixed rapidly into 450 $mm^3$ of a hypotonic medium containing detergent, RuBP and $NaH^{14}CO_3$ as described by Robinson, McNeil, and Walker (1979). After 1 min incubation at 20 °C, 100 $mm^3$ 2 N HCl was added. $^{14}C$, incorporated into acid stable compounds, was determined. ATP in extracts was measured by the luciferase method using the luminometer and assay chemicals from LKB Wallac (Turku, Finland). RuBP was measured in the same extracts by the method of Latzko and Gibbs (1974).

### *Purification of RuBPCO*

The first leaves of 10 d old wheat seedlings (50 g) were homogenized in 100 $cm^3$ of 20 mM Tris HCl (pH 8·0), 10 mM mercaptoethanol, 1·0 mM EDTA, and $(NH_4)_2SO_4$ giving 37% saturation in the homogenate. The homogenate was filtered through cheese cloth and the filtrate centrifuged at 20 000 × *g* for 20 min. $(NH_4)_2SO_4$ was added to the supernatant to give 50% saturation. After 10 min centrifugation at 10 000 × *g* the supernatant liquid was decanted and the precipitate dissolved in extraction buffer (6·0 $cm^3$). The solution was applied to a chromatography column (25 mm × 500 mm) of Sephadex G-25 (course), equilibrated with extraction buffer. The eluate was applied to a column (25 mm × 1000 mm) of Sepharose Cl-6B, equilibrated with 5·0 mM Hepes KOH (pH 8·0), and 1·0 mM dithiothreitol. Eluted fractions containing high RuBPCO activity were combined and freeze-dried.

### *Experiments with purified RuBPCO*

A sample of freeze-dried enzyme was dissolved in distilled water and the solution mixed with an equal volume of 200 mM Tris HCl (pH 8·6) containing 40 mM $MgCl_2$ and 20 mM $NaHCO_3$ and activated by incubation at 40 °C for 20 min. Activated enzyme was diluted 1:10 with 100 mM Tris HCl (pH 8·0) and 5·0 mM dithiothreitol giving concentrations of 1·0 mM $NaHCO_3$ and 2·0 mM $MgCl_2$. Effectors were added as indicated in Table 1 and solutions incubated for 20 min at 20 °C. 50 $mm^3$ samples were tested for RuBPCO activity in 0·5 $cm^3$ assay medium at 20 °C as described by Lorimer, Badger, and Andrews (1977).

## RESULTS

### *1. RuBPCO activity in chloroplasts with and without $P_i$ in the medium*

Chloroplasts were pre-incubated in the dark at 20 °C with 5·0 mM $P_i$ or without $P_i$. After 20 min, the suspensions were illuminated and samples taken at intervals for RuBPCO activity tests (Fig. 1), RuBPCO activity increased for 4 min. This increase was relatively independent of the $P_i$ concentration in the medium. Thereafter, RuBPCO activity decreased considerably if the medium was deficient in $P_i$, but only slightly in the presence of 5·0 mM $P_i$. $O_2$ evolution was very low in both treatments. The results suggest that $P_i$ does not affect RuBPCO activity

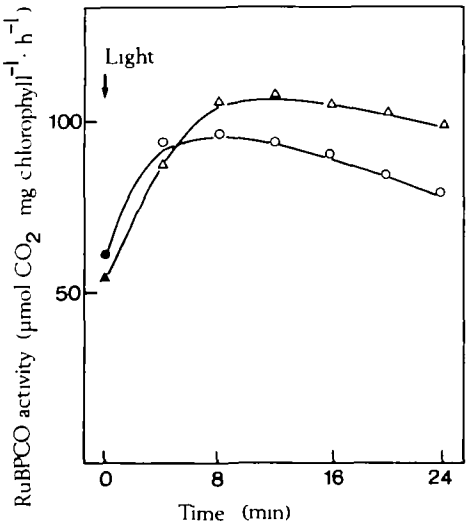


FIG. 1. RuBPCO activity in chloroplasts with and without $P_i$ in the medium. Chloroplasts (28 $\mu g$ chlorophyll $cm^{-3}$ suspension) were pre-incubated in the dark at 20 °C in 50 mM Hepes KOH (pH 7·6), 0·4 M sorbitol, 1·0 mM $NaHCO_3$, 1·0 mM EDTA and 200 units catalase $cm^{-3}$ suspension with either 5·0 mM $P_i$ (△) or without $P_i$ (○). After 20 min pre-incubation, chloroplasts were illuminated and samples taken at intervals for measurement of RuBPCO activity. Closed symbols represent measurements in the dark, before illumination.

in chloroplasts in the dark and during induction of photosynthesis after illumination. However, $P_i$ seems to prevent RuBPCO from inactivation when photosynthesis proceeds.

*2. The ATP and RuBP contents of illuminated chloroplasts as influenced by $P_i$ and the relationship to RuBPCO activity*

Chloroplasts were pre-incubated for 20 min in the dark without $P_i$ and then illuminated (Fig. 2). After a few minutes of photosynthesis 0·0, 0·3, or 5·0 mM $P_i$ was added. Oxygen evolution was measured. Samples were taken at intervals and tested for RuBPCO activity and ATP concentration. RuBPCO activity and ATP decreased during photosynthesis in $P_i$ deficient medium, but increased after addition of $P_i$. The increase was sharper with 5·0 mM $P_i$ than with 0·3 mM $P_i$. $O_2$ evolution was low with no $P_i$ and with 5·0 mM $P_i$, but it was high with 0·3 mM $P_i$. The results suggest that the decrease in RuBPCO activity could be related to the decrease in ATP concentration.

The ATP concentration and its relation to RuBP was investigated during photosynthesis by chloroplasts at various $P_i$ concentrations and at 5 °C or 25 °C. ATP and RuBP concentrations were determined after the release of 2·5 $\mu$mol $O_2$ $mg^{-1}$ chlorophyll (Table 1). When the $P_i$ concentration in the medium was too low (no $P_i$ added at 5 °C) or too high (1·8 mM $P_i$ at 25 °C), the rate of photosynthesis was not high enough to release 2·5 $\mu$mol $O_2$ $mg^{-1}$ chlorophyll (Mächler *et al.*, 1984). This was due to an imbalance between $CO_2$ assimilation and assimilate export. RuBP increased and ATP decreased when chloroplasts seemed to be deficient in $P_i$. This occurred at 25 °C in the absence of $P_i$ and at 5 °C with 0·2 mM $P_i$. The increased RuBP under $P_i$ deficient conditions seems to be the result of decreased RuBPCO activity, although it may be due partly to the influence of accumulated metabolites on the equilibrium of the exergonic RuBPCO reaction. The increase in RuBP when ATP decreased supports the hypothesis of a relationship between RuBPCO activity and ATP concentration.

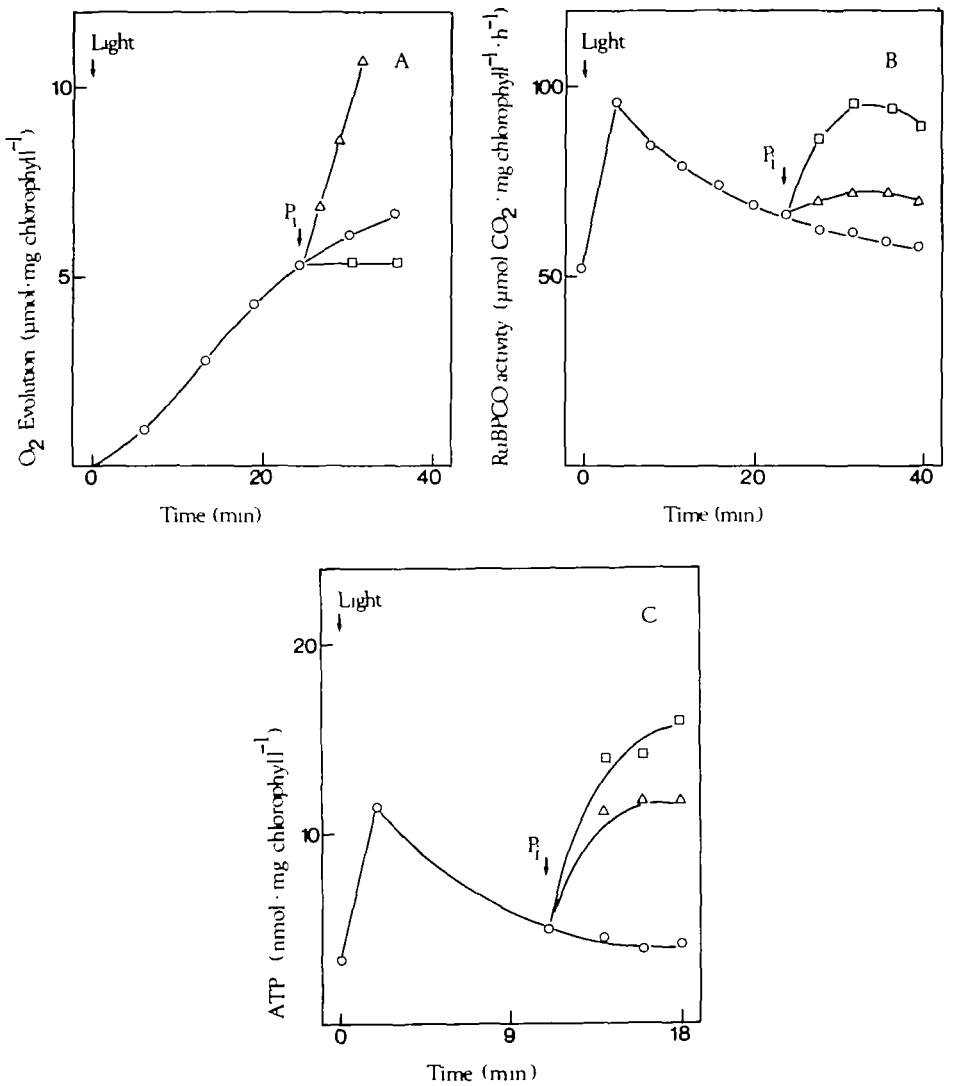


FIG. 2. Photosynthetic $O_2$ release (A), RuBPCO activity (B), and ATP content (C) of illuminated chloroplasts as influenced by $P_i$. Chloroplasts (20 $\mu$g chlorophyll cm$^{-3}$ suspension) were pre-incubated and illuminated as described in Fig. 1 without $P_i$ (○). After a period of photosynthesis 0·3 mM $P_i$ (△), 5·0 mM $P_i$ (□), or no $P_i$ (○) was added. Samples were taken at intervals and tested for RuBPCO activity and ATP content. Oxygen evolution was monitored.

### *3. RuBPCO activity in chloroplasts during the photoreduction of various substrates differing in the consumption of ATP and $P_i$*

Chloroplasts were pre-incubated in the dark for 20 min at 20 °C without $P_i$ in 1·0 mM $NaHCO_3$ and with or without one of the following substrates: 1·0 mM oxaloacetate, 1·0 mM 3-phosphoglycerate, or 1·0 mM glycerate, and then illuminated (Fig. 3). The photoreduction of oxaloacetate, 3-phosphoglycerate, glycerate, and $CO_2$ was calculated to require 0, 2, 4, and 3 mol ATP and 0, 0, 2, and 1 mol $P_i$ per mol $O_2$ released. RuBPCO was activated during the induction lag of photosynthesis when $CO_2$, oxaloacetate, or glycerate were the substrates. Thereafter, RuBPCO was inactivated during the photoreduction of $CO_2$ and glycerate but not

TABLE 1. *ATP and RuBP content of chloroplasts illuminated at 5 °C or 25 °C at various $P_i$ concentrations*

Chloroplasts (60 μg chlorophyll $cm^{-3}$ suspension) were pre-incubated in the dark for 20 min at 25 °C or for 40 min at 5 °C at various $P_i$ concentrations and with 1·0 mM $NaHCO_3$ and then illuminated. 100 $mm^3$ samples of the chloroplast suspensions were taken when 2·5 μmol $O_2$ had been released, mixed immediately with 20 $mm^3$ of 3 N $HClO_4$ and centrifuged for 10 min at 3000 × *g*. 20 $mm^3$ of 1·0 M $K_2HPO_4$ was added to 100 $mm^3$ of supernatant and pH adjusted to 7·2 by adding 18 $mm^3$ of 3 N KOH. $KClO_4$ was sedimented and ATP and RuBP in the supernatant determined.

| $P_i$ in the medium | nmoles RuBP / mg chlorophyll | | nmoles ATP / mg chlorophyll | |
|---|---|---|---|---|
| | 5 °C | 25 °C | 5 °C | 25 °C |
| No $P_i$ in medium | — | 23·9 | — | 2·4 |
| 0·2 mM $P_i$ | 19·4 | 14·4 | 3·6 | 6·6 |
| 0·6 mM $P_i$ | 14·9 | 14·6 | 6·2 | 7·4 |
| 1·8 mM $P_i$ | 13·4 | — | 8·6 | — |

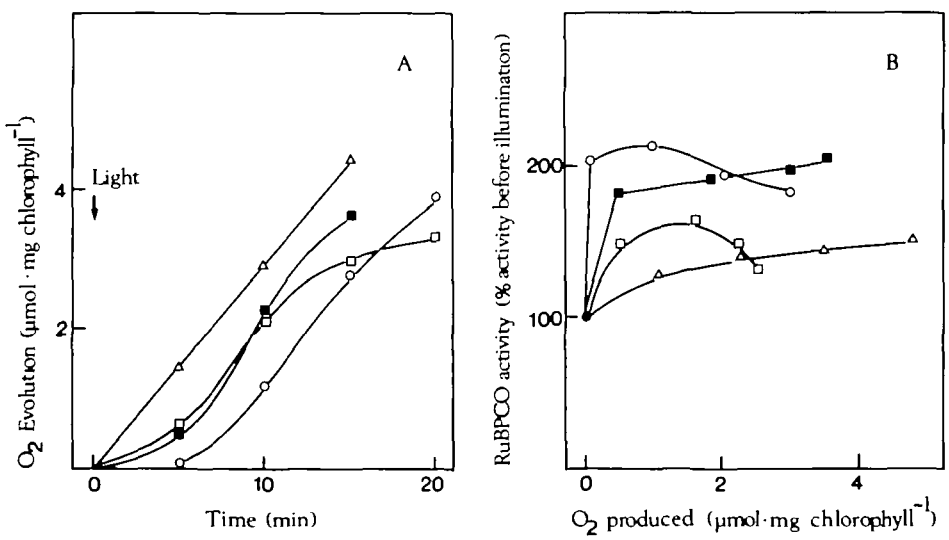


FIG. 3. Photosynthetic $O_2$ release (A) and RuBPCO activity (B) in chloroplasts during the photoreduction of various substrates differing in the consumption of ATP and $P_i$. Chloroplasts (90 μg chlorophyll $cm^{-3}$ suspension) were pre-incubated in the dark for 20 min at 20 °C in 50 mM Hepes KOH (pH 7·6), 0·4 M sorbitol, 1·0 mM $NaHCO_3$, 1·0 mM EDTA and 200 units catalase $cm^{-3}$ suspension with 1·0 mM oxaloacetate (■), 1·0 mM 3-phosphoglycerate (△), 1·0 mM glycerate (□) or no additional substrate (○). Chloroplasts were illuminated and samples taken for measurement of RuBPCO activity. RuBPCO activity before illumination was 26, 32, 23, and 31 μmol $CO_2$ $mg^{-1}$ chlorophyll $h^{-1}$ for oxaloacetate, 3-phosphoglycerate, glycerate, and no additional substrate, corresponding to 100%. RuBPCO activity was plotted against the sum of $O_2$ released.

during the photoreduction of oxaloacetate. The photoreduction of $CO_2$ and glycerate is associated with ATP and $P_i$ consumption, whereas for the photoreduction of oxaloacetate neither ATP nor $P_i$ is required. The results suggest a relationship between inactivation of RuBPCO and the consumption of ATP and $P_i$. Only decreased activation of RuBPCO occurred upon illumination in the presence of 3-phosphoglycerate, a substrate without

induction lag of photosynthesis. The photoreduction of 3-phosphoglycerate needs ATP, whereas no $P_i$ is consumed. The decreased RuBPCO activity during photoreduction of 3-phosphoglycerate seems to be related to a decreased ATP concentration. RuBPCO activity was decreased, although no $P_i$ was consumed suggesting that RuBPCO activity is related to ATP and not to $P_i$ directly.

*4. Purified RuBPCO as influenced by $P_i$ or other metabolites when ATP is present*

The inconsistency between the activation of RuBPCO by $P_i$ *in vitro* and the lack of a direct relationship between $P_i$ and RuBPCO activity *in vivo* was the incentive for the following experiments with purified RuBPCO.

Purified RuBPCO was pre-incubated in the presence of various compounds for 20 min at 20 °C and tested for RuBPCO activity (Table 2). RuBPCO was activated by $P_i$, 3-phosphoglycerate, and NADPH and inactivated by ribose-5-phosphate regardless of whether ADP was present or not. However, when ATP was present, RuBPCO was neither activated by $P_i$ nor by 3-phosphoglycerate and only slightly by NADPH. Inactivation by ribose-5-phosphate was reinforced by ATP. ATP alone seemed to decrease RuBPCO activity to a lesser extent than when it was combined with a metabolite. The ATP effect seemed not to be due to sequestration of free $Mg^{++}$ since it occurred not only with 2·0 mM $Mg^{++}$ but also with 20 mM $Mg^{++}$, although to a lesser extent. The results suggest that the increased RuBPCO activity in chloroplasts in the presence of increased ATP and $P_i$ concentrations is not due to direct effects of these compounds on the enzyme.

TABLE 2. *Influence of $P_i$ and other metabolites on the activity of purified RuBPCO in the presence and absence of ATP and ADP*

Purified RuBPCO was incubated in 100 mM Tris HCl (pH 8·0), 1·0 mM $NaHCO_3$, 2·0 or 20·0 mM $MgCl_2$, 5·0 mM dithiothreitol, and effectors, as indicated in the table, for 20 min at 20 °C. RuBPCO activity was tested at 20 °C by adding 50 $mm^3$ of pretreated enzyme to 450 $mm^3$ of assay mixture. (n.d. = not determined, 3-PGA = 3-phosphoglycerate, R-5-P = ribose-5-phosphate).

| Effectors added | RuBPCO activity ($\mu$moles $CO_2$ $min^{-1}$ $mg^{-1}$ protein) | | | | |
|---|---|---|---|---|---|
| | 2·0 mM $Mg^{++}$ | | | 20·0 mM $Mg^{++}$ | |
| | Control | 2·0 mM ATP | 2·0 mM ADP | 2·0 mM ATP | 2·0 mM ADP |
| Zero | 0·162 | 0·130 | n.d. | n.d. | n.d. |
| 2·0 mM $P_i$ | 0·289 | 0·126 | 0·264 | 0·762 | 0·836 |
| 2·0 mM 3-PGA | 0·275 | 0·145 | 0·238 | 0·696 | 0·780 |
| 2·0 mM R-5-P | 0·096 | 0·043 | 0·088 | 0·197 | 0·364 |
| 2·0 mM NADP | n.d. | 0·140 | 0·252 | 0·466 | 0·592 |
| 2·0 mM NADPH | n.d. | 0·205 | 0·432 | 0·861 | 0·880 |

## DISCUSSION

Inactivation of RuBPCO during photosynthesis seems to be related to a decreased $P_i$ concentration in the stroma. RuBPCO activity seems to decrease when $P_i$ in the stroma is consumed. RuBPCO is reactivated when $P_i$ is added to the chloroplast suspension. However, $P_i$ neither increases RuBPCO activity in the dark nor during the induction lag of photosynthesis upon illumination.

In illuminated chloroplasts ATP content, $P_i$ concentration and RuBPCO activity seem to increase and decrease correspondingly. ATP seems to prevent $P_i$ from activating RuBPCO

and thus a direct activation of RuBPCO by $P_i$ is unlikely. The study suggests that RuBPCO activity is more closely related to ATP than to $P_i$.

It has been suggested earlier (Mächler *et al.*, 1984) that the ratios of ATP/ADP and dihydroxyacetone phosphate/3-phosphoglycerate decrease in illuminated chloroplasts when $P_i$ is decreased. The decrease in RuBPCO activity, which occurs correspondingly, cannot be explained by a direct effect of these compounds on the enzyme. However, the pH in the stroma may decrease due to decreased ATP and accumulated 3-phosphoglycerate. This may be responsible for enzyme inactivation.

$P_i$ deficiency in the chloroplast stroma occurs when $CO_2$ assimilation exceeds assimilate export. Photosynthetic products accumulate as organic phosphates in the stroma, resulting in decreased stromal $P_i$ (Mächler *et al.*, 1984). The decrease in RuBPCO activity and photosynthesis, which occurs under these conditions, thus appears as a feedback inhibition.

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